Amendments to the Specification:

Please add the following new paragraph to the specification as the first paragraph of the specification following the title of the invention.

This application is a continuation of U.S. Patent Application Serial No. 09/564,559, filed May 4, 2000, which is a continuation-in-part of U.S. Patent Application Serial No. 09/023,809, filed February 13, 1998, now U.S. Patent No. 6,183,738, which claims the benefit of U.S. Provisional Application No. 60/046,200, filed May 12, 1997, each of which is hereby incorporated by reference in its entirety.

Please delete the paragraph beginning at line 15 of page 2 and ending at line 26 of page 2 and replace it with the following new paragraph:

Certain disadvantages have come to be associated with the isolation of arginine deiminase from organisms. Although effective in killing tumor cells *in vitro*, arginine deiminase isolated from *Pseudomonas pudita* failed to exhibit efficacy *in vivo* because it had little enzyme activity at a neutral pH and was rapidly cleared from the circulation of experimental animals. Arginine deiminase derived from *Mycoplasma arginini* (SEQ ID NO:5) is described, for example, by Takaku et al, *Int. J. Cancer, 51*:244-249 (1992), and U.S. Patent No. 5,474,928, the disclosures of which are hereby incorporated by reference herein in their entirety. A problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of arginine deiminase from *Mycoplasma arginini*, via a cyanuric chloride linking group, with polyethylene glycol was described by Takaku et al., *Int. J. Cancer Res.* 84:1195-1200 (1993). The modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group.

Please delete the paragraph beginning at line 27 of page 2 and ending at line 7 of page 3 and replace it with the following new paragraph:

The production of arginine deiminase via recombinant DNA techniques also provides for certain disadvantages. For example, arginine deiminase produced in [*Escheria*] *Escherichia coli* is enzymatically inactive and thus must be denatured and then properly renatured in order for it to become enzymatically active. The usual method for renaturing arginine deiminase produced in *E. coli* is to isolate the inactive enzyme, dissolve it in [guanidium] guanidinium hydrochloride and renature it by rapid dilution into low ionic strength buffer. This last step requires very large volumes of buffer thus making the manufacture of arginine deiminase both expensive and time consuming. However, recombinant technology does have certain advantages. For example, organisms more amenable to fermentation can be used as hosts. Additionally, these fermentation hosts are generally much less pathogenic and larger amounts of arginine deiminase can be obtained. It has been shown the *E. coli* may produce large amounts of *Mycoplasma* arginine deiminase.

Please delete the paragraph beginning at line 9 of page 4 and ending at line 10 of page 4 and replace it with the following new paragraph:

Figure 1 depicts the amino acid sequence of arginine deiminase cloned from wild-type *Mycoplasma hominus* (SEQ ID NO:1).

Please delete the paragraph beginning at line 11 of page 4 and ending at line12 of page 4 and replace it with the following new paragraph:

Figure 2 depicts the amino acid sequence of modified arginine deiminase from *Mycoplasma hominus* in accordance with preferred embodiments of the present invention (SEQ ID NO:4).

Please delete the paragraph beginning at line 24 of page 5 and ending at line 9 of page 6 and replace it with the following new paragraph:

The amino acid sequences of arginine deiminase from the *Mycoplasma hominus* gene is disclosed by Figures 1 (SEQ ID NO:1) or 2 (SEQ ID NO:4). Chemical and genetic modification of the arginine deiminase enzyme can affect its biological activities. For example, it has been shown that arginine deiminase is typically antigenic and rapidly cleared from circulation in a patient. However, it has also been shown that the formulation of arginine deiminase with polyethylene glycol reduces the antigenicity and increases the circulating half-life of the enzyme. Abuchowski et al., *Cancer Biochem. Biophys.* 7:175-186 (1984); Abuchowski et al., *J. Biol. Chem.* 252:3582-3586 (1977). In particular, arginine deiminase can be covalently modified with polyethylene glycol. Arginine deiminase covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG." In U.S. Patent Application Serial No. 09/023,809, Clark describes improved modifications of arginine deiminase from *Mycoplasma hominus* (SEQ ID NO:1), *Mycoplasma arginini* (SEQ ID NO:5), and *Mycoplasma arthritides* (SEQ ID NO:7) with polyethylene glycol, the disclosure of which is hereby incorporated by reference herein in its entirety. When compared to native arginine deiminase, ADI-PEG retains most of its enzymatic activity, is far less antigenic, has a greatly extended circulating half-life, and is much more efficacious in the treatment of tumors. For purposes of the invention, the modification of any arginine deiminase with polyethylene glycol may be referred to as pegylation.

Please delete the paragraph beginning at line 6 of page 8 and ending at line 4 of page 9 and replace it with the following new paragraph:

It is to be understood that arginine deiminase derived from other organisms may also have pegylation sites corresponding to 112 position of arginine deiminase from *Mycoplasma hominus*. For example, arginine deiminase from *Steptococcus pyrogenes* has lysine at the 104 position, arginine deiminase from *Mycoplasma pneumoniae* has lysine at the 106 position, and arginine deiminase from *Qiardia intestinalis* has lysine at the 114 position. In addition, arginine deiminase from some organisms may have lysines corresponding to the same general location as the 112 position of arginine deiminase from *Mycoplasma hominus*. The location of lysine in arginine deiminase from such organisms may be indicated as follows:

Table 1: Pegylation sites of arginine deiminase from various organisms

Organisms producing arginine deiminase	**Position of lysine in arginine deiminase**
Mycoplasma hominus (SEQ ID NO:1)	112
Mycoplasma arginini (SEQ ID NO:5)	111
Clostridium perfringens	105
Bacillus licheniformis	97, 108
Borrelia burgdorferi	102, 111
Borrelia afzellii	101
Enterococcus faecalis	102, 110
Streptococcus pyogenes	104

Steptococcus pneumoniae	103
Lactobacillus sake	97, 106
Qiardia intestinalis	114, 116

It is presently believed that the attachment of polyethylene glycol to such lysines or combinations thereof may inactivate the enzyme. It is presently believed that amino acid substitutions at such lysines may result in a protein that loses less of its enzymatic activity upon pegylation.

Please delete the paragraph beginning at line 5 of page 9 and ending at line 25 of page 9 and replace it with the following new paragraph:

The present invention thus provides for certain amino acid substitutions in the polypeptide chain of arginine deiminase. These amino acid substitutions provide for modified arginine deiminase that loses less activity upon pegylation; i.e. upon pegylation, the reduction of enzyme activity following pegylation in the modified arginine deiminases is less than the reduction of enzyme activity following pegylation in the unmodified arginine deiminases. By eliminating pegylation sites at or adjacent to the catalytic region of enzyme, optimal pegylation can be achieved without the traditional loss of activity. As discussed above, arginine deiminase from certain organisms have pegylation sites located at various positions on the peptide chain. While not limiting the present invention, it is presently believed that arginine deiminase may have the amino acid lysine located at or adjacent to the catalytic region of the enzyme and that pegylation of these sites may inactivate the enzyme. By eliminating at least one of these pegylation sites, pegylation can be achieved and more

enzyme activity retained. In accordance with the invention, it is preferred that lysine is substituted with glutamic acid, valine, aspartic acid, alanine, isoleucine, leucine or combinations thereof. More preferred is that lysine is substituted with glutamic acid. In one embodiment of the invention, it is preferred that modified arginine deiminase from *Mycoplasma hominus* has an amino acid substitution at Lys^{112}, Lys^{374}, Lys^{405}, Lys^{408} or combinations thereof. Preferably, modified arginine deiminase from *Mycoplasma hominus* has an amino acid substitution Lys^{112} to Glu^{112}, Lys^{374} to Glu^{374}, Lys^{405} to Glu^{405}, Lys^{408} to Glu^{408} or combinations thereof. More preferred is that modified arginine deiminase from *Mycoplasma hominus* has lysine at position 112 substituted with glutamic acid (SEQ ID NO:2).

Please delete the paragraph beginning at line 13 of page 10 and ending at line 3 of page 11 and replace it with the following new paragraph:

The present invention thus again provides for certain amino acid substitutions in the polypeptide chain of arginine deiminase. Such amino acid substitutions can eliminate the problematic structural characteristics in the peptide chain of arginine deiminase. Such amino acid substitutions provide for improved renaturation of the modified arginine deiminase. These amino acid substitutions make possible rapid renaturing of modified arginine deiminase using reduced amounts of buffer. These amino acid substitutions may also provide for increased yields of renatured modified arginine deiminase. In one embodiment of the invention, it is preferred that the modified arginine deiminase have a single amino acid substitution at Pro^{210}. As mentioned above, arginine deiminase derived from *Mycoplasma hominus* has the amino acid proline located at the 210 position. While not limiting the present invention, it is presently believed that the presence of the amino acid proline at

position 210 results in a bend or kink in the normal polypeptide chain that increases the difficulty of renaturing (i.e., refolding) arginine deiminase. Substitutions for proline at position 210 make possible the rapid renaturation of modified arginine deiminase using reduced amounts of buffer. Substitutions for proline at position 210 may also provide for increased yields of renatured modified arginine deiminase. In a preferred embodiment, the proline at position 210 is substituted with serine (SEQ ID NO:3). It is to be understood that in accordance with this aspect of the invention, other substitutions at position 210 may be made. Examples of preferred substitutions include Pro^{210} to Thr^{210}, Pro^{210} to Arg^{210}, Pro^{210} to Asn^{210}, Pro^{210} to Gln^{210} or Pro^{210} to Met^{210}. By eliminating those structural characteristics associated with the amino acid of position 210 of the wild-type arginine deiminase, proper refolding of the enzyme can be achieved.

Please delete the paragraph beginning at line 4 of page 11 and ending at line 18 of page 11 and replace it with the following new paragraph:

In another embodiment of the invention, it is preferred that the modified arginine deiminase have multiple amino acid substitutions. The modified arginine deiminase may have at least one amino acid substitution eliminating pegylation sites at or adjacent a catalytic region of the enzyme. The modified arginine deiminase may also have at least one amino acid substitution eliminating those structural characteristics that interfere with the renaturation of the enzyme. The amino acid substitutions may thus provide for a modified arginine deiminase of the invention. The amino acid substitutions may provide for the pegylation of modified arginine deiminase without a loss of enzymatic activity. The amino acid substitutions may provide for a modified arginine deiminase that can be rapidly renatured using reduced amounts of buffer. The amino acid substitutions may also provide

for increased yields of renatured modified arginine deiminase. In a preferred embodiment, the modified arginine deiminase derived from *Mycoplasma hominus* includes the proline at position 210 substituted with serine and the lysine at position 112 substituted with glutamic acid (SEQ ID NO:4). As discussed above, however, it is to be understood that the modified arginine deiminase may include other preferred substitutions.

Please delete the paragraph beginning at line 12 of page 16 and ending at line 15 of page 16 and replace it with the following new paragraph:

Modified arginine deiminase was expressed in JM101 cells as previously described by Takaku et al., *supra*. The modified arginine deiminase included glutamic acid at the 112 position and serine at the 210 position. The amino acid sequence of modified arginine deiminase from *Mycoplasma hominus* is described in Figure 2 (SEQ ID NO:4).

Please delete the paragraph beginning at line 18 of page 16 and ending at line 2 of page 17 and replace it with the following new paragraph:

The modified arginine deiminase (SEQ ID NO:4) was isolated and purified as previously described by Takaku et al., *supra*. However, several improvements were observed.

Table 2: Renaturation of Arginine Deiminase

Compound	Time (° C)	Dilution Ratio	Yield
Arginine Deiminase (SEQ ID NO:1)	90 hours (15 ° C)	1:200	70 mg/L

Modified Arginine Deiminase (SEQ ID NO:4)	6-12 hours (room temp.)	0.0763889	500 mg/L

As indicated by Table 2 above, renaturation of the modified arginine deiminase was completed at room temperature in about 6 to 12 hours using a 1:50 dilution ratio of guanidium hydrochloride inclusion bodies in buffer. In contrast, Takaku et al. reported that renaturation required 90 hours at 15 °C using a 1:200 dilution ratio. In addition, the yield of modified arginine deiminase was routinely about 500 mg per liter of fermentation whereas Takaku et al. reported a yields of approximately 70 mg per liter of fermentation.

Please delete the paragraph beginning at line 4 of page 17 and ending at line 14 of page 17 and replace it with the following new paragraph:

Modified arginine deiminase (SEQ ID NO:4) was formulated using SS-PEG as previously described. The pegylation process was allowed to go unchecked for over 4 hours without the modified arginine deiminase becoming inactivated. In addition, it was not necessary to quench the pegylation process through the addition of glycine. With reference to Table 3, approximately 70-80% of enzymatic activity of the modified arginine deiminase was retained.

Table 3: Specific Enzyme Activity (IU/mg protein)

	Without Pegylation	**Pegylated**
wild type ADI (SEQ ID NO:1)	20-21	36653
modified ADI	20-21	36875

(SEQ ID NO:4)

This indicates that the modified arginine deiminase allows for more consistent formulations. In addition, the modified arginine deiminase allows for scaling up of the manufacturing process as compared to the wild-type arginine deiminase.